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(54) METHOD OF AUTOMATED CLASSIFICATION OF  
MICRONUCLEATED ERYTHROCYTES,  
DEVICE FOR AUTOMATED CLASSIFICATION OF  
MICRONUCLEATED ERYTHROCYTES, AND  
METHOD OF MAKING MICRONUCLEATED ERYTHROCYTE SPECIMEN

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## Specification

### 1. Title of the Invention

Method of automated classification of micronucleated erythrocytes, device for automated classification of micronucleated erythrocytes, and method of making micronucleated erythrocyte specimen.

### 2. Scope of Patent Claim

(1) A method of automated classification of micronucleated erythrocytes, wherein the enlarged image of erythrocytes of a specimen is introduced to a video camera with optical filters of each of the three colors of red, green and blue; blue-green image data, which are the difference between the blue and green, and green-red image data, which are the difference between the green and red, are each processed from the red, green, and blue image data that have been introduced; the above-mentioned green data are converted to binary values and the erythrocytes and background are separated into regions; a concentration histogram of each video data is made; this erythrocyte region is further separated into the micronucleus part and the reticulocyte cytoplasm part, the feature parameters of each separate region are calculated by color theory processing and the feature parameters pertaining to cell shape are calculated from the above-mentioned image data; and branch theory processing is performed based on each of these feature parameters.

(2) A device for automated classification of micronucleated erythrocytes, comprising a microscope with which an enlarged view of a specimen is obtained, a video camera to which the enlarged image of the specimen obtained with this microscope is introduced using optical filters in the 3 colors of red, green and blue; an analog/digital converter, with which analog/digital conversion of the red, green and blue signals of this video camera is performed, a memory means in which these red, green and blue image data are stored, a video data processing means that processes the blue-green video data, which are the difference between the blue and green, and the green-red video data, which are the difference between green and red; a first region separation means, which converts the green image data stored in said memory means to binary data and separates the erythrocytes and background into regions; a histogram drafting means, which drafts a concentration histogram of each video data stored in said memory means and the video data processed by said video data processing means; a second region separation means, which further separates [the data] into the micronucleus part and reticulocyte cytoplasm part based on the histogram drafted by said histogram drafting means; a feature parameter calculation means, which calculates the feature parameters for each region that has been separated by this second region separation means by color theory processing and calculates the feature parameters pertaining to the shape of the cell from the video data stored in said memory means and the video data processed by said video data processing means; and a branching theory processing means with which erythrocytes having a micronucleus are classified by branching theory processing based on the feature parameters calculated by this feature parameter calculation means.

(3) A method of making a micronucleated erythrocyte specimen, whereby blood is passed through a column packed with  $\alpha$ -cellulose/microcrystalline cellulose mixture so that cells other than erythrocytes will be adsorbed on the above-mentioned  $\alpha$ -cellulose/microcrystalline cellulose mixture, this column is washed with bovine fetal serum and the effluent is centrifuged to trap the erythrocytes, ultrasensitive biological staining of these erythrocytes is performed using new methylene blue, a centrifuged smear of the erythrocytes that have been submitted to ultrasensitive biological staining is made on slide gas and the centrifuged smear of erythrocytes is Wright stained.

### 3. Detailed Description of the Invention

#### (a) Industrial field of use

The present invention pertains to a method of automated classification of micronucleated erythrocytes, an automated classification device suitable for this automated classification method, and further, a method of making specimens suitable for this automated classification method.

#### (b) Prior art

A micronucleus is a piece of cell nucleus that is produced as a result of chromosomal aberration induced by exposure to radioactivity, chemical substances, etc. When the micronucleation mechanism in erythrocytes is explained, erythrocytes are produced by bone marrow and when they are produced, they have a nucleus and are called erythroblasts. The erythroblasts become polychromatic erythrocytes (PCE) having large amounts of RNA as a result of denucleation by cellular division and maturation. The amount of RNA decreases and the erythrocytes are released peripherally as

normochromatic (NC) by further maturation. If anomalies are produced in chromosomes for some reason or another during the stage of division of this erythroblast, a fragment is produced as a result of cleavage during the intermediate stage. This fragment does not change, even during the last stage [of cell division], and it appears as a micronucleus separate from the daughter nuclei beginning with [this] last stage [of cell division].

The micronucleus test is a test that is used to detect chromosomal aberration induced by the above-mentioned chemical substances and radioactivity, and it is widely used as a mutagenicity screening test. Conventional methods involve smearing blood cells of rodents on a slide glass (slide smear) and observing these blood cells under a microscope in order to classify the micronucleated polychromatic erythrocytes and find their incidence.

(c) Problems to be solved by the invention

The above-mentioned smear specimens comprise erythrocytes, as well as leukocytes, platelets, dust, etc., and because the micronucleus is extremely small, training is required in order to classify cells under a microscope. Moreover, the incidence of cells with a micronucleus is very low and it therefore takes considerable time and effort to perform the micronucleus test by the above-mentioned visual classification.

Furthermore, it is said that a minimum of 4,000 cells must be counted because this incidence is so low, but only 1,000 cells can be observed from 1 animal for the above-mentioned reasons.

The present invention is offered in light of the above-mentioned, its purpose being to present a method of automated classification of micronucleated erythrocytes, and an

automated classification device and method of making specimens suitable for this automated classification method.

(d) Means for solving problems and effects

The method of automated classification of micronucleated erythrocytes in claim 1 for solving the above-mentioned problem has the structure described under the following i ~ vii:

- i: the enlarged image of erythrocytes of a specimen is introduced to a video camera with optical filters in each of the three colors of red, green and blue,
- ii. blue-green image data, which are the difference between the blue and green, and green-red image data, which are the difference between the green and red, are each processed from the red, green, and blue image data that have been introduced,
- iii. the above-mentioned green data are converted to binary values and the erythrocytes and background are separated into regions,
- iv. a concentration histogram of each video data is made,
- v. this erythrocyte region is further separated into the micronucleus part and the reticulocyte cytoplasm part based on the histogram that was made,
- vi. the feature parameters of each separate region are calculated by color theory processing and the feature parameters pertaining to cell shape are calculated from the above-mentioned image data, and
- vii. branch theory processing is performed based on each of these feature parameters.

The method of automated classification of micronucleated erythrocytes of claim 1 emphasizes the fact that the red, green and blue transmission factors of erythrocytes and

[those] of micronuclei and reticulocyte cytoplasm in the erythrocytes are [either] very different or are similar in the respective wavelength region. Moreover, the erythrocytes and background are separated based on the fact that absorption by erythrocytes in the green wavelength region is strong. Moreover, the blue-green video data and green-red video data are calculated and the difference between the erythrocytes, micronuclei and reticulocyte cytoplasm is enhanced so that the micronuclei and reticulocyte cytoplasm can be easily separated into regions.

On the other hand, the branching theory is applied to the feature parameters obtained from the concentration histogram and video data. This branching theory is characterized in that it is similar in structure to the human judgment theory and expert knowledge can be easily incorporated into the theory so that feature parameters and judgment criteria coincide 1:1, making structural modification of the theory simple. Consequently, automated classification of micronucleated erythrocytes that is just as accurate as visual classification becomes possible.

The device for automated classification of micronucleated erythrocytes in claim 2 has the following structure described in the following i ~ x:

- i: a microscope with which an enlarged view of a specimen is obtained,
- ii. a video camera to which the enlarged image of the specimen obtained with this microscope is introduced using optical filters in the three colors of red, green and blue,
- iii. an analog/digital converter, with which analog-digital conversion of each of the red, green and blue signals of this video camera is performed,
- iv. a memory means in which these red-green and blue image data are stored,

v. a video data processing means that processes the blue-green video data, which are the difference between blue and green, and the green-red video data, which are the difference between green and red,

vi. a first region separation means, which converts the green image data stored in said memory means to binary data and divides erythrocytes and background into regions,

vii. a histogram drafting means, which drafts a concentration histogram of each video data stored in said memory means and the video data processed by said video data processing means,

viii. a second region separation means, which further separates [the data] into the micronucleus part and reticulocyte cytoplasm part based on histograms drafted by said histogram drafting means,

ix. a feature parameter calculation means, which calculates the feature parameters for each region that has been separated by this second region separation means by color theory processing and calculates the feature parameters pertaining to the shape of the cell from the video data stored in said memory means and the video data processed by said video data processing means, and

x. a branching theory processing means with which erythrocytes having a micronucleus are classified by branching theory processing based on the feature parameters calculated by this feature parameter calculation means.

This automatic classification device in claim 2 is suitable for the automatic classification method of claim 1 and is made so that the image signals of the video camera are digitally converted and processing, such as video data processing, region separation, etc., are performed by digital processing.



The method of making micronucleated erythrocytes specimens of claim 3 is suitable for the automated classification method in claim 1 and has the structure described in the following i ~ v:

- i. blood is passed through a column packed with  $\alpha$ -cellulose/microcrystalline cellulose mixture so that cells other than erythrocytes will be adsorbed on the above-mentioned  $\alpha$ -cellulose/microcrystalline cellulose mixture,
- ii. this column is washed with bovine fetal serum and the effluent is centrifuged to trap the erythrocytes,
- iii. ultrasensitive biological staining of these erythrocytes is performed using new methylene blue,
- iv. a centrifuged smear of these erythrocytes that have been submitted to ultrasensitive biological staining is made on slide glass and
- v. the centrifuged smear of erythrocytes is Wright stained.

By means of this method of making specimens of claim 3, other cells can be removed with an  $\alpha$ -cellulose/microcrystalline cellulose mixture and therefore, it is not necessary to perform video data treatment in order to separate the erythrocytes and other cells into regions at the type of automatic classification and classification of erythrocytes with micronuclei can be simplified.

(e) Examples

An example of the present invention will be described below in Figures 1 through 9.

Figure 1 is a block diagram describing the device for automated classification of micronucleated erythrocytes. 2 is the automated microscope for obtaining an enlarged

image of the specimen. A photograph of this enlarged image of this automated microscope 2 can be taken by video camera 3. Moreover, it can be taken with color video camera 5 and sensor 14 using beam splitters 4a and 4b.

Sensor 14 output is input to autofocus circuit 15 and signals are further output to focus drive circuit 16 and focus of automated microscope 2 is adjusted automatically so that the focus matches that of autofocus circuit 15.

Stage drive circuit 17 drives the stage of automated microscope 2. Moreover, autofeeder controller 18 has a function for successive introduction of specimens S to this stage.

These autofocus circuit 15, focus drive circuit 16, stage drive circuit 17, and autofeeder controller 18 are all controlled by CPU 10.

On the other hand, color video camera 5 holds interference filters (not illustrated) in three colors, that is, red (R), green (G), and blue (B). Video signals of each color of R, G, and B are output. The wavelength region transmitted by these interference filters is shown in Figure 3. The transmission factor of micronuclei and erythrocytes to wavelength is also shown in Figure 3. Interference filters having a wavelength region having wavelengths mainly in the wavelength region wherein there is a large difference in the [micronucleus and erythrocyte] transmission factors and in the wavelength region wherein the [micronucleus and erythrocyte] transmission factors are close to one another are used in order to enhance the color information of the cells.

Video signals (R, G, B) of this color video camera 5 are also incorporated into the above-mentioned CPU 10. However, they are digitally converted by analog/digital (A/D) converter 6 and stored in video memory 7. By means of this example, color video

camera 5 converts each R, G and B color to digital signals at 6 bits (64 gradation) with the 51.2 x 38.4  $\mu\text{m}$  center of vision of microscope 2 serving as the photographing region and sampling pitch being 0.1  $\mu\text{m}$  horizontal and vertical.

Feature extraction processor 8 is connected to video memory 7. This feature extraction processor 8 has a function for making R-B video data and B-G video data, a function for separating erythrocytes and background into regions, a function for making a concentration histogram, and a function for calculation of feature parameters.

Recognition processor 9 is further connected to feature extraction processor 8 and has a function for classification of erythrocytes using the branching theory for the feature parameters that have been calculated by feature extraction processor 8.

Video memory 7, feature extraction processor 8, and recognition processor 9 are all controlled by CPU 10.

Monitor 11, such as a CRT, etc., keyboard 12, and printer 13, are further connected to CPU 10. Monitor 11 is for monitoring the video image obtained from color TV camera 5 and video data, histogram, classification results, etc. Printer 13 also prints these classification results. Keyboard 12 is for inputting each type of command.

Next, making the specimens for this automated classification device will be described while referring to Figure 6. 21 in Figure 6 is a syringe that is used as a column.  $\alpha$ -cellulose/microcrystalline cellulose mixture 22 is packed inside syringe 21.

This  $\alpha$ -cellulose/microcrystalline cellulose mixture 22 is packed inside syringe 21 after dispersing (1 hour at room temperature) the equivalent amount (g) in fetal cow serum (FCS). Column capacity is 2 ml. Moreover, the filter paper at the tip of syringe 21 is so that  $\alpha$ -cellulose and microcrystalline cellulose do not flow out [of the syringe].

1 to 2 ml peripheral blood 24 to which EDTA has been added as anticoagulant is passed through syringe 21 from the top. The leukocytes and platelets in this blood 24 are adsorbed on  $\alpha$ -cellulose/microcrystalline cellulose mixture 22.

The inside of this syringe 21 is further washed several times with FCS (2 ml at a time) and the effluent is centrifuged to trap the erythrocytes. 99% of the leukocytes and 88% of the platelets are removed by this operation to obtain erythrocytes almost exclusively.

Next, ultrasensitive biological staining of these erythrocytes is performed with new methylene blue and the reticulocyte cytoplasm in the polychromatic erythrocytes is selectively colored. Moreover, centrifugation and smearing on slide glass, Romanovsky's staining, and in the present example, Wright staining are performed.

Automated calculation using the specimen made in this way will be described below while referring to Figures 2, 4, 5, 7 through 9, etc.

First, a specimen was introduced to the stage of automated microscope 2 by autofeed controller 18 and [the microscope was focused] [Step (hereafter referred to as ST) 1]. The enlarged image of the specimen photographed by video camera 3 in this state is shown in Figure 4. The erythrocytes shown with arrows in Figure 4 have micronuclei.

Next, the enlarged image of this specimen is introduced to color video camera 5 and the R, G and B video signals are converted to digital signals by A/D converter 6 (ST2). Feature extraction processor 8 cuts out a 128 x 128 video region for each of the R, G and B digital signals and makes digital image data in each of the 3 colors of R, G and

B and R-B (difference between red signals and blue signals) [digital video data] and B-G (difference between blue signals and green signals) [digital video data] (ST3).

Next, feature extraction processor 8 makes binary signals from the G signals, which show the strongest absorption by normochromatic erythrocytes (NCE), in order to separate the background from the video data (ST4).

$$\text{NCE} = \{ (x,y) \mid G(x,y) < \alpha \}$$

x : integer with which  $1 \leq x \leq 128$

y: integer with which  $1 \leq y \leq 128$

G(x,y): G concentration of pixels (x,y)

(provided that  $0 \leq G(x,y) \leq 63$ )

$\alpha$ : extraction fixed threshold

Even if fixed  $\alpha$  is used as the threshold for binary value conversion, there will be a very large concentration difference between the background and erythrocytes and stable region separation becomes possible regardless of the target specimen.

Feature extraction processor 8 makes a histogram of the R, G, B, R-B and B-G concentrations of the erythrocyte regions in order to separate the erythrocyte part from the adhesion part (micronucleus, reticulocyte cytoplasm, platelets, dust, etc.)

As shown in Figure 5, the micronuclei and reticulocyte cytoplasm present in the erythrocytes differ from erythrocytes and dust, etc., in terms of their R-B and B-G signals. Using this feature, region separation into micronuclei and reticulocyte cytoplasm is performed by the mode method based on the R-B and B-G concentration histogram. Furthermore, of the concentration histograms obtained in this way, Figure 7 shows the R-

B concentration histogram. The region to the left of  $\alpha$  corresponds to reticulocyte cytoplasm and the region to the left of  $\beta$  corresponds to micronuclei.

At ST6, feature extraction processor 8 performs color theory processing on the separated regions, calculates the feature parameters pertaining to micronucleus, reticulocyte cytoplasm, and erythrocyte concentration and color, and calculates feature parameters relating to the shape of the cells, such as volume, peripheral length, shape, number of clumps, etc., from the shape of the cells. Furthermore, these basic parameters are combined and secondary feature parameters are calculated.

The feature parameters used in the present example are listed below:

(1) Shape information of concentration histogram (refer to Figure 7)

1. Length of concentration histogram (LX, LN)

Each color of R, G, B, R-B, B-G

2. Length of concentration histogram of region ( $L\alpha$ ,  $L\beta$ )

Each color of R-B, B-G

(2) Concentration information

1. Average concentration of blood cells (DNS)

Each color of R, G, B, R-B, B-G

2. Average concentration of region ( $DNS\alpha$ ,  $DNS\beta$ )

Each color of R-B, B-G

(3) Color information

1. Average concentration

Each color of R, G, B, R-B, B-G

(4) Surface area information

1. Blood cell surface area (NA)

Each color of R, G, B

2. Region surface area ( $NA\alpha$ ,  $NA\beta$ ) (refer to Figure 7)

- (5) Shape information

1. Erythrocyte outside peripheral shape (SHP)

Each color of R, G, B

2. Region shape ( $SHP\alpha$ ,  $SHP\beta$ )

Each color of R-B, B-G

Distribution of several feature parameters will be described. The micronucleus shows little absorption of B signals and very strong absorption of R and G signals.

[Blood components] can be classified as polychromatic erythrocytes with a micronucleus (MNPCE), erythrocytes with a micronucleus (MNNCE), polychromatic erythrocytes (PCE), normochromatic erythrocytes (NCE), and others based on the distribution of the length of the concentration histogram [LX(R-B), LX (B-G)], which is the feature parameter that uses this [absorption pattern].

[Blood components] can be generally classified as polychromatic erythrocytes with a micronucleus (MNPCE), normochromatic erythrocytes with a micronucleus (MNNCE), and polychromatic erythrocytes (PCE) based on the distribution of the region surface area  $NA\alpha$  and  $NA\beta$  (refer to Figures 8(B) and (c)).

Although not illustrated, [blood components] can also be classified as those in which micronucleus, reticulocyte cytoplasm, or micronucleus and reticulocyte cytoplasm adhere to the inside of the erythrocyte or other based on the shape parameters of the separated regions.

In ST7, recognition processor 9 compares [the results] with the judgment criteria for each blood cell by applying the branching theory to the above-mentioned feature parameters and thereby differentiates between cell components. The cells are classified by repeating this [procedure]. Classification is into the 5 types of polychromatic cell with micronucleus (MNPCE), normochromatic erythrocyte with micronucleus (MNNCE), polychromatic erythrocyte (PCE), erythrocyte with other adhesion (OTHER), and normochromatic erythrocyte (NCE).

In ST8, these classification results are displayed on monitor 11 and printed out by printer 13.

Next, studies will be performed on reliability of automated classification of micronucleated erythrocytes in the present example.

The data used in the studies were from 74 blood cells input from 5 specimens (17 MNPCE, 5 MNNCE, 21 PCE, 11 OTHER and 20 NCE), and classification was by an expert panel.

Agreement between the experts and automated classification of each blood cells is represented by a confusion matrix (refer to Figure 9). That is, how each 1 cell was differentiated was investigated and represented as the specific statistical amount for the automated classification versus the expert judgment. The figures with which the classification agreed are shown in the diagonal matrix from the left to the right, with the number of blood cells that coincide with one another shown at the top and the percentage agreement (represented by %) shown at the bottom.

Percentage agreement =



(Number of cells on which there was agreement between experts and automated classification/Number judged by experts) x 100.

As shown in Figure 9, it was possible to confirm that the percentage agreement in terms of the micronucleus was 100% for MNPCE and MNNCE, which is satisfactory detection reliability. Moreover, there were no instances of false recognition of micronuclei and satisfactory results were also obtained in terms of the percentage correct. However, there were 2 examples wherein other adhesions were falsely identified (specifically, dust from the stain) in identification of polychromatic erythrocytes.

(f) Results of the invention

As previously explained, the method of automated classification of micronucleated erythrocytes in claim 1 has an advantage in that labor and time needed for the micronucleus test can be reduced. Moreover, it has an advantage in that many cells can be observed and therefore, micronucleus test reliability can be improved.

Moreover, the device for automated classification of micronucleated erythrocytes in claim 2 has an advantage in that video signals are digitally converted and region separation, feature parameter processing, branching theory implementation, etc., are digitally performed, simplifying the automated classification method of claim 1.

Furthermore, the method of making micronucleated erythrocytes in claim 3 has an advantage in that leukocytes and platelets are removed from blood and micronuclei and reticulocyte cytoplasm are selectively colored by ultrasensitive biological staining, facilitating the automated classification method of claim 1.

#### 4. Brief Description of the Figures

Figure 1 is a block diagram of the device for automated classification of micronucleated erythrocytes shown in an example of the present invention, Figure 2 is a flow chart describing operation of the same device for automated classification of micronucleated erythrocytes, Figure 3 is a diagram showing the properties of the interference filter used in the same device for automated classification of micronucleated erythrocytes, Figure 4 is a diagram of one example of an enlarged view of a specimen, Figure 5 is a diagram describing the video signals when erythrocytes are scanned so that they are cut horizontally, Figure 6 is a diagram describing the method of making a specimen used for the above-mentioned device for automated classification of micronucleated erythrocytes, Figure 7 is a diagram showing an example of a concentration histogram made using the same device for automated classification of micronucleated erythrocytes, Figure 8(a), Figure 8(b), and Figure 8(c) are figures describing the distribution of feature parameters calculated by the same device for automated classification of micronucleated erythrocytes, and Figure 9 is a diagram showing the confusion matrix of a device for automatic classification of micronucleated erythrocytes.

2. Automated microscope
5. Color video camera
6. A/D converter
7. Video memory
8. Feature extraction processor
9. Recognition process
21. Syringe

## 22. $\alpha$ -cellulose/microcrystalline cellulose mixture

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Figure 1.

- 5. Color video camera
- 7. Video memory
- 8. Feature extraction processor
- 9. Recognition processor
- 11. Monitor
- 12. Keyboard
- 13. Printer
- 14. Sensor
- 15. Autofocus
- 16. Focus drive circuit
- 17. Stage drive circuit
- 18. Autofeeder controller

Figure 2.

Start

ST1. Specimen set

ST2. Photography

ST3. Making of video data

ST4. Binary conversion of G fide data, background separation

ST5. Making of histogram

ST6. Feature parameter calculation

ST7. Classification by branching theory

ST8. Result calculation

End

Figure 3.

Key: a. Transmission factor (%)

b. Wavelength (nm)

c. B filter

d. G filter

e. R filter

f. Micronucleus

g. Erythrocyte

Figure 4.

Figure 5.

Key: a. Erythrocyte  
b. Reticulocyte cytoplasm  
c. Dust  
d. Micronucleus

Figure 6.

Key: 21. Syringe  
22.  $\alpha$ -cellulose/microcrystalline cellulose mixture

Figure 7.

Key: a. Frequency

Figure 8(a)

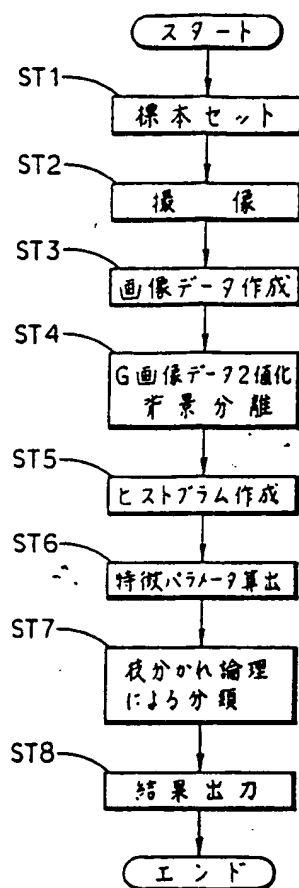
Figure 8(b)

Figure 8(c)

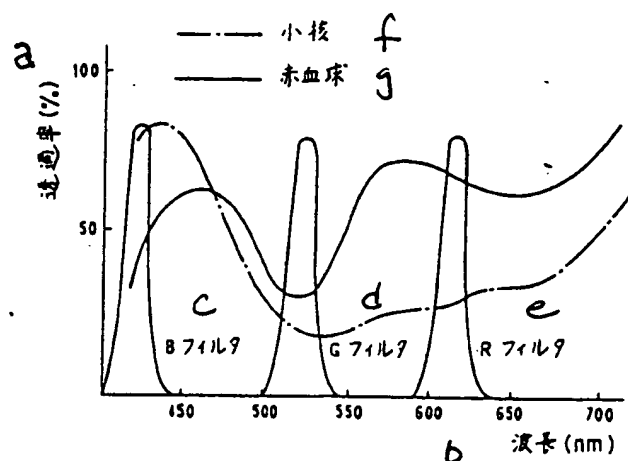
Figure 9.

Key: a. Automated classification  
b. Visual classification

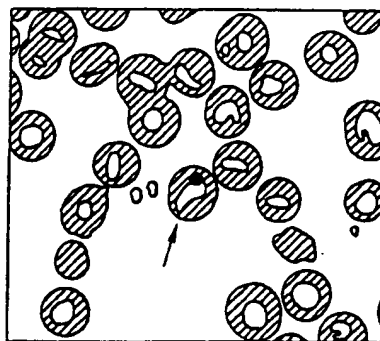
第 2 図

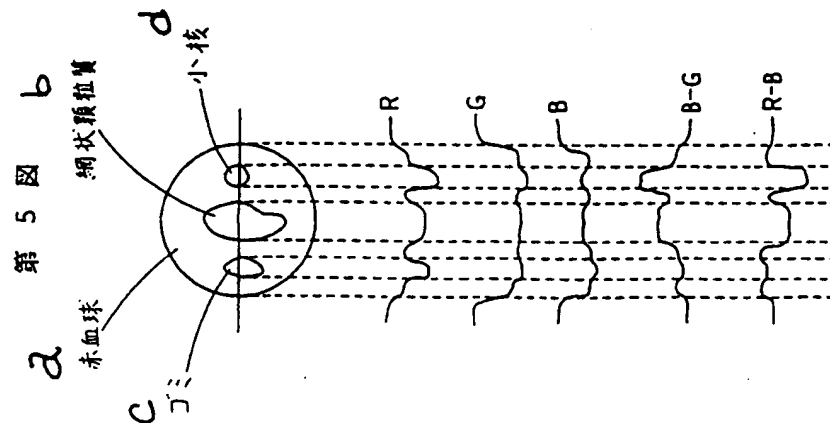


第 3 図

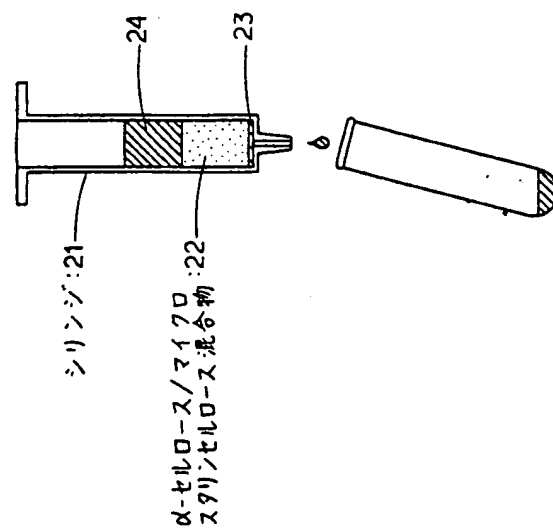


第 4 図

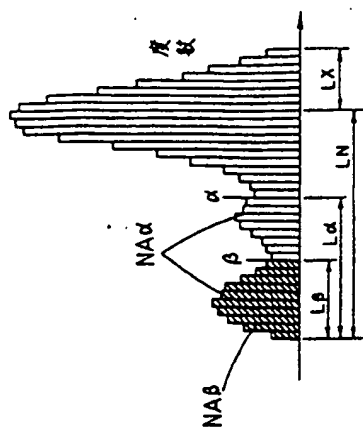




第 6 図



第 7 図



第 8 図 (a)

